TRYPTOPHAN–HEME ENERGY TRANSFER IN HUMAN HEMOGLOBIN: DEPENDENCE UPON THE STATE OF THE IRON

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Received 4 March 1980
Revised version received 28 May 1980

1. Introduction

In hemoglobin the apoprotein conformation interacts reversibly with the heme and affects the ligand binding behavior [1]. The conformational changes produced in the protein matrix by ligand binding have been characterized in the crystal [2]. Aromatic amino acid residues, particularly tryptophan, are intrinsic protein fluorophores which are often very sensitive to their local environment [3]. Human hemoglobin contains several tryptophan residues which may potentially serve as intrinsic probes even though their fluorescence yields are extremely poor [4]. Recently the emission spectra and relative quantum yields of tryptophan in hemoglobin and its isolated subunits were determined [5]. These investigations, however, did not extend to a study of the coupling interaction between the heme and the protein matrix.

This note reports the utilization of intrinsic tryptophan fluorescence to study the dynamics of the apoprotein moiety upon oxygen or carbon monoxide binding. The results demonstrate that binding of ligands to deoxyhemoglobin affects the transfer of excitation energy from the aromatic amino acid residues to the heme moieties. The results also suggest a statistical fluctuation in the coupling between the fluorophors (tryptophan residues) and their quenchers (heme residues).

2. Materials and methods

Hemoglobin A was extracted from fresh human adult blood by standard procedures [6]. The solution was purified and desalted through a mixed-bed ion exchange resin (AG 501-X8, Bio-Rad) [7]. For all experiments, 0.1 M phosphate buffers with a minimum fluorescence background were prepared from water purified by a millipore filtration system. Equilibration with gasses (N_2, O_2, CO) and spectrophotometric measurements were done at 25°C. The absence of methemoglobin in the sample was verified by the constancy of the absorbance over pH 6–8.5. The effects of ligand binding upon tryptophan emission were measured under 1 atm. gas. Fluorescence measurements were performed with Suprasil cuvettes, scrupulously cleaned, of 1 cm pathlength sealed to tonometers. Emission spectra were obtained upon excitation at 280 nm; all spectra were obtained with 2 nm excitation and emission bandwidths. The exciting light was the synchrotron radiation from the electron storage ring, ACO (Anneau de Collisions D'Orsay) [8]. Emission spectra were obtained on a photon counting spectrofluorometer interfaced to a Nuclear Data 660 acquisition system which is built around an LSI11 microcomputer [9] (fig.1). For lifetime measurements the same apparatus was used except that the Nuclear Data 660 was operated in the pulse height analysis mode. The most important advantages of the synchrotron radiation over conventional pulsed sources for lifetime determinations are the temporal pattern of the pulse (narrow, ~1.2 ns and gaussian shape), the invariance of pulse shape with wavelength and the high repetition rate, ~13.6 MHz. The high repetition rate allows for good counting statistics even for the weak emissions observed in the present study. A_{280} values were maintained at 0.05 to minimize inner filter effects and trivial absorption of the fluorescence by heme

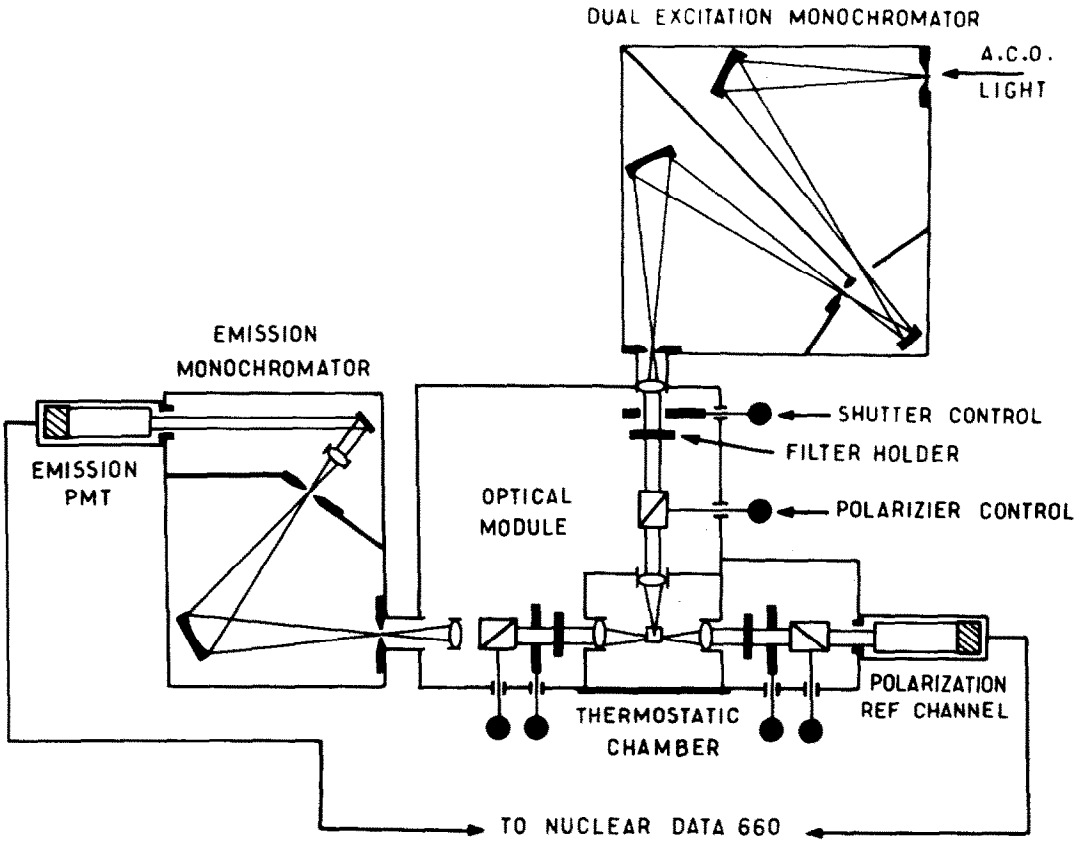

Fig.1. Scheme of the spectrofluorometer coupled to the synchrotron radiation from ACO.

moieties. The buffer background consisting of the water Raman peak, contaminant fluorescence and dark counts were subtracted from all fluorescence measurements. The relative constancy of the ACO pulse shape and amplitude with time greatly facilitates the subtraction of background counts in the lifetime measurements.

3. Results and discussion

The emission spectra obtained at pH 7, shown in fig.2, demonstrate that the emission maxima are the same and the halfwidths approximately constant for the 3 forms studied: deoxyhemoglobin, HbO_2 and HbCO. Note that the apohemoglobin emission spectrum (excited at 280 nm) is also very similar to these spectra [10]. The spectral shape and bandwidths were invariant over pH 6–8.5.

The results indicate that the tryptophan residues in deoxyhemoglobin and its various liganded forms (and also in apohemoglobin) are exposed to the same kind of relatively non-polar or non-relaxing microenvironment [11]. Differences in the emission maxima which could be correlated to conformational changes were not observed.

The quantum yields obtained for HbCO were systematically lower (30%) than those for HbO_2. This difference, outside the limit of error (10%) may be due to a perturbation of Förster-type energy transfer [12] from tryptophan to heme. Modification of the tryptophan–heme orientation is a probable explanation. The fluorescence polarization spectra of HbCO and deoxyhemoglobin are the same as that found for HbO_2 [5]. The large polarization upon excitation at 300 nm observed for all these proteins, compared to the intrinsic limiting polarization for tryptophan, indicates that the tryptophans do not move appreciably during the lifetime of the excited state.

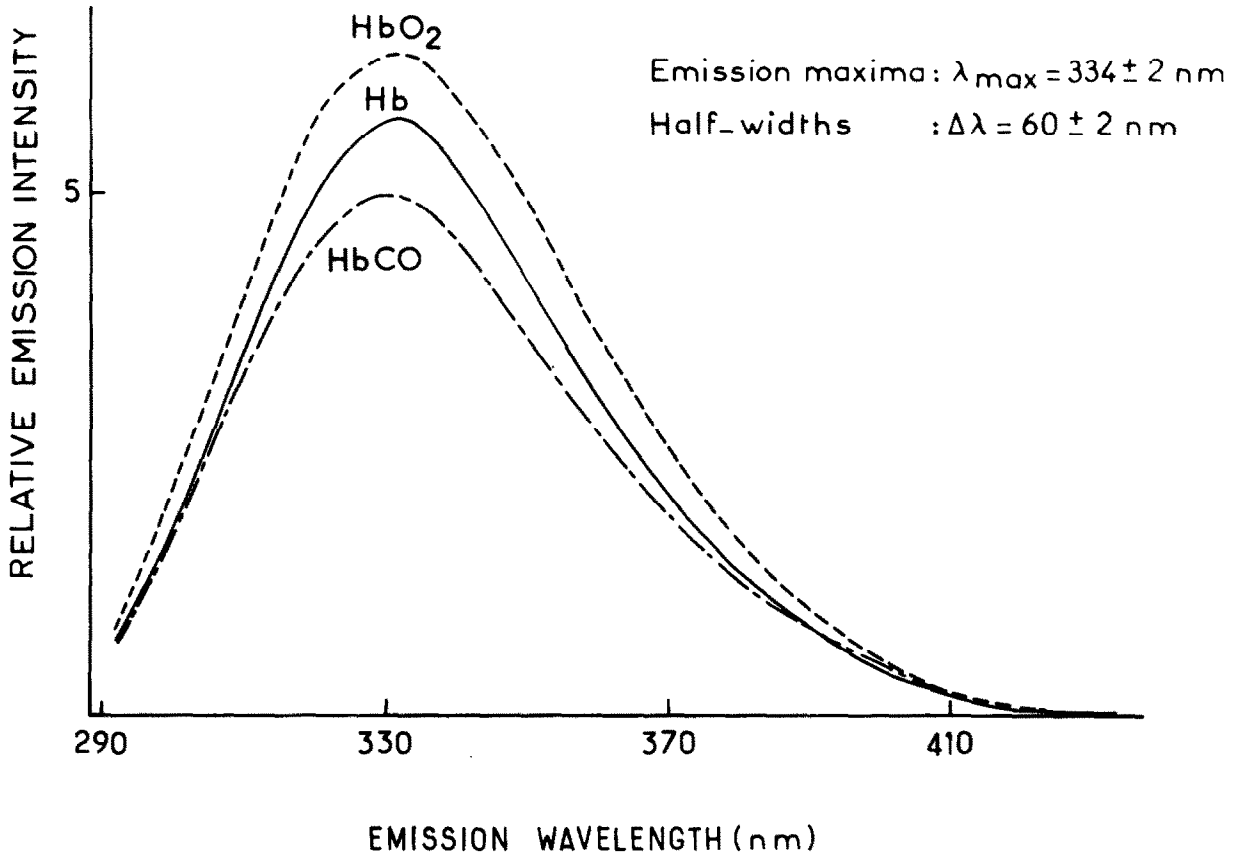

Fig.2. Corrected emission spectra of human hemoglobin, obtained at 25°C with 280 nm excitation wavelength. The A_{280} values of the 3 hemoglobin forms were ~0.05. The 0.1 M phosphate buffer was at pH 7. Quantum efficiencies relative to tryptophan at 25°C were: 1.20% (Hb); 1.35% (HbO_2); 1.0% (HbCO); error limit, 10%.

No differences were apparent in the decay times of the tryptophan emission from deoxyhemoglobin and HbCO. There was, however, a small but significant decrease in the quantum yield when deoxyhemoglobin became liganded with CO. This observation suggests that the quenching may be related to some form of a 'static' complex [13] between tryptophan and heme. We should note here, though, that little work has been done to date on the effects of heterotransfer upon fluorescence lifetimes and that the exact relationship between the intensity decrease and lifetime decrease in such a complex system is not clearly understood. The observed hemoglobin fluorescence decay curves are multiexponential (fig.3) and a complete analysis in terms of component lifetimes and amplitudes does not seem feasible at this time. A major component would seem to be ~1.5 ns, however, a value anomalously long compared to the poor quantum yield; anomalously long lifetimes for various hemoglobins have been reported [14]. The different emitting species probably reflect the effects of collective movements (fluctuations in the tryptophan–heme orientation) [14–16] within the macromolecule. The energy transfer coupling between tryptophan and heme is evidently not permanent and the oscillation times of tryptophan residues may be slow compared to the lifetime of the excited state. The relaxation processes for excited tryptophan follow complicated kinetics and the emitted light probably originates statistically from a small fraction of the total excited tryptophan population. The observations on the lifetimes and the quantum yields suggest that specific quenching processes for each iron ligand do not exist but that the oscillation time

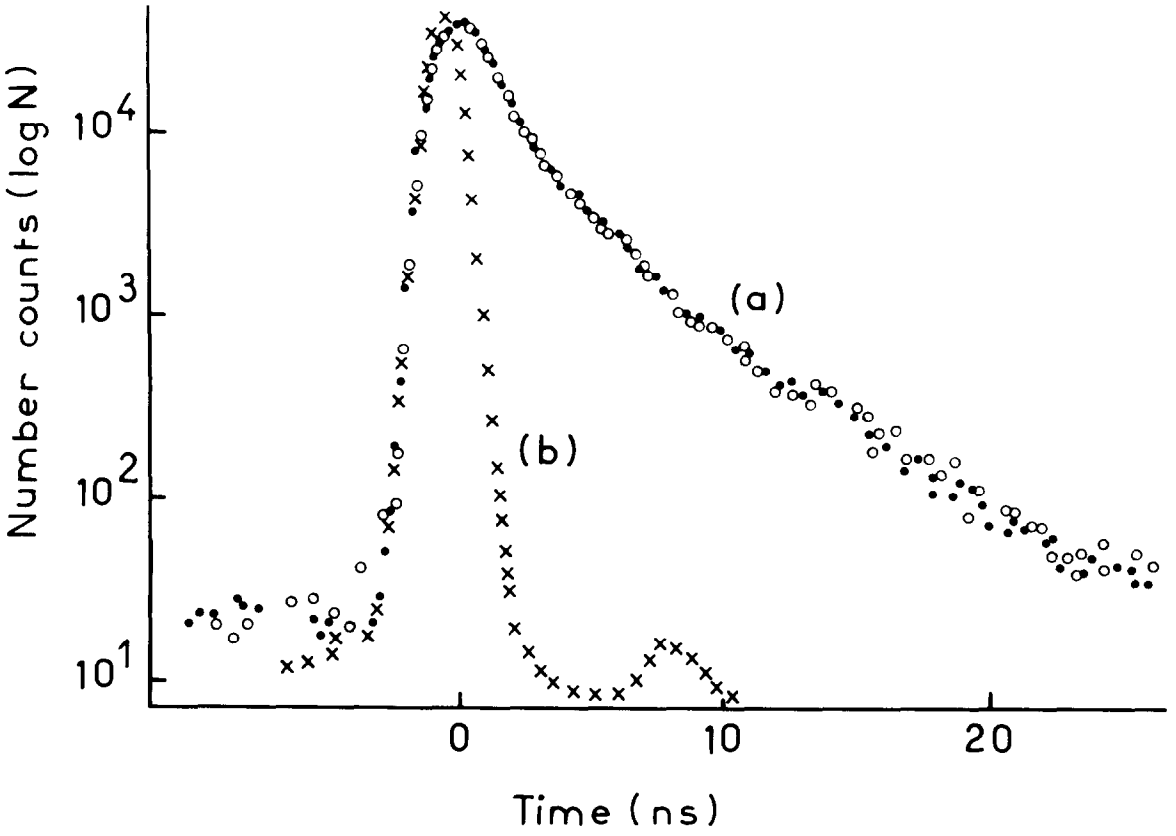

Fig.3. Fluorescence decay for human hemoglobin, obtained at 25°C with 280 nm excitation wavelength; emission observed at 340 nm; all slits were 4 nm. (a) (• •) HbCO; (○ ○) Hb, in 0.1 M phosphate buffer at pH 7; (b) (× ×) ACO pulse.

of the tryptophan–heme couple depends upon the ligand.

Simple consideration of the distance between the center of the heme (Fe) and tryptophan A_{12} (in α or β chains) and C_3 (in β chain) based on the data in [2] would suggest that tryptophan C_3, at 5 Å from the heme, would be totally quenched. It may be, however, that, as regards tryptophan C_3, an equilibrium exists between hemoglobin conformations permitting transfer and those restricting transfer. Relative orientations and motions of groups involved in energy transfer mechanisms may dramatically affect the transfer efficiency and we may expect that the observed, i.e., unquenched emission, may reveal subtle changes in the globin structure on dynamics. Further work, particularly on time-dependent polarization and time-resolved emission and excitation spectra could serve to establish a dynamics picture of these hemoproteins.

Acknowledgements

We thank Professor G. Weber for his interest in this work. We are very much indebted to technicians and engineers of Orsay Linear Accelerator Laboratory for running ACO for synchrotron radiation. This work was supported in part by the Institut National de La Santé et de la Recherche Médicale (contrat 78.1.059.5) and by an Action Thématique Programmée of the CNRS (ATP 3117) for building the fluorometer coupled to ACO.

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